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**THE EFFECTS OF GAIN OF FUNCTION MUTANT P53 AND P63 ON EPS8
AND CXCL5 EXPRESSION IN HEAD AND NECK SQUAMOUS CELL
CARCINOMA**

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Abstract

THE EFFECTS OF GAIN OF FUNCTION MUTANT P53 AND P63 ON EPS8 AND CXCL5 EXPRESSION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.
Virginia Commonwealth University, 2013.

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Head and neck squamous cell carcinoma (HNSCC) is one of the ten most common cancers worldwide, with a survival rate of less than 50%. A class of mutant p53, known as gain of function (GOF) mutant p53, has been found to be expressed in tumors in these patients. GOF mutant p53 not only loses the wild type tumor suppressor functions, but also gains aberrant functions that have been linked to tumorigenesis. In this current study, we utilized a model system consisting of cells derived from HNSCC tumors in order to investigate our hypothesis that GOF mutant p53 enhances and p63 inhibits EPS8 and CXCL5 expression and promoter activity. We found decreased EPS8 expression, CXCL5 expression, and cellular migration associated with the loss of GOF mutant p53. This indicates an enhancing role of GOF mutant p53 in cellular migration and expression of these target genes. The loss of GOF mutant p53 was also associated with decreased EPS8 and CXCL5 promoter activity, indicating upregulation of these target gene promoters by GOF mutant p53. We found increased EPS8 expression, CXCL5 expression, and cellular migration with the loss of p63 in cell expressing high levels of p63. This indicates an

inhibitory role of p63 on the expression of these target genes and cellular migration. Loss of p63 was also associated with increased EPS8 and CXCL5 promoter activity, indicating p63 may be downregulating these target gene promoters. EPS8 and CXCL5 in tumorigenesis, our findings suggest that GOF mutant p53 and p63 play role in tumorigenesis. Additional studies are needed to further elucidate the mechanism by which GOF mutant p53 and p63 regulate EPS8 and CXCL5 expression and promoter activity.

Introduction

1.1 Head and Neck Squamous Cell Carcinoma

Head and neck squamous cell carcinoma (HNSCC) encompasses cancers deriving from the mucosal surfaces of the head and neck including the oral cavity, pharynx, larynx, paranasal sinuses and nasal cavity¹. HNSCC is one of the ten most common cancers worldwide, with over 650,000 new cases reported annually. In the United States, HNSCC accounts for about 3% of reported malignancies². The overall survival rate is low at 50%, which can be attributed to late presentation and metastatic tumor progression³.

Risk factors for HNSCC include tobacco use (smoking or chewing), heavy alcohol use, human papilloma virus (HPV) and poor oral hygiene. Tobacco use is a major risk factor due to carcinogens present causing genotoxic stress that can lead to the mutations and the formation of tumors. Acetaldehyde, a metabolite of alcohol, interferes with DNA synthesis and repair. The effects of alcohol are synergistic with tobacco, while heavy alcohol consumption is also recognized as an independent risk factor. Oral HPV infection has been shown to be linked to HNSCC², with evidence mounting for an etiological role of HPV infection, specifically the HPV E6 oncoprotein, in HNSCC⁴. Current therapies for HNSCC include surgery, chemotherapy, radiation therapy, or a combination of therapies and treatment plans that vary by location of primary tumor and clinical stage. HPV positive patients also receive differing treatment therapies than those who are HPV negative¹.

1.2 Tumor Metastasis

The oral cavity is lined by stratified squamous epithelia delineated by a continuous basement membrane. The basement membrane regulates differentiation and migration of epithelial cells and also serves as a barrier to invasion during tumorigenesis (Figure 1). Tumors arise through progressive acquisition of genetic alterations affecting regulation of cell growth, motility, and stromal interactions due to exposure to carcinogens. Prior to invasion, abnormal cellular growth, differentiation, and stratification occurs in the epithelium².

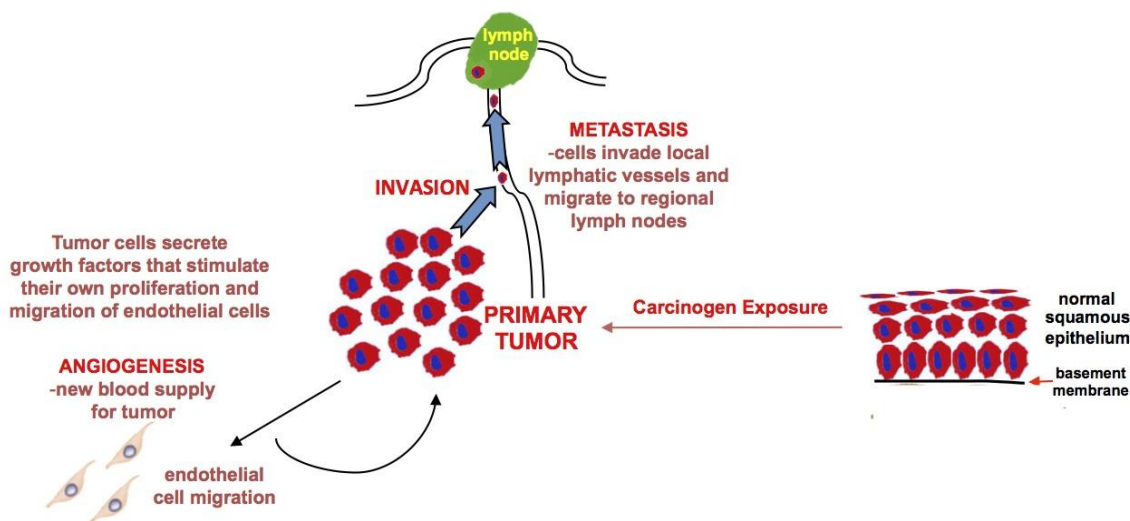


Figure 1 Tumor Progression and Metastasis in HNSCC. Squamous epithelium transforms into an invasive tumor from exposure to carcinogens. These tumor cells stimulate development of their own blood supply and cellular growth and migration. Migration and invasion can lead to secondary tumor growth, known as metastasis. Modified from Philips Institute Webpage⁵.

Primary HNSCC tumors are aggressive locally and often metastasize to nearby lymph nodes and less often to distant sites³. Metastasis occurs when cancer cells invade local tissues and move into lymph vessels or blood vessels (Figure 1). Cancer cells are

able to move through the lymphatic system or bloodstream to distant sites in the body where they can migrate into surrounding tissues. At distant locations, cancer cells can proliferate and stimulate angiogenesis⁶. Invasion and metastasis occur when tumor cells acquire distinct phenotypes from the primary tumor cells including increased motility, ability to degrade surrounding extracellular matrix (ECM), and enhanced survival. Cytokines and growth factors play a key role in cell migration and invasion during metastasis³.

1.3 p53

p53 is a transcription factor involved in cell cycle regulation, apoptosis, and angiogenesis encoded by the gene TP53. The p53 protein binds in a sequence specific manner to target genes involved in growth arrest, DNA damage repair, apoptosis and inhibition of angiogenesis. Normally, p53 is kept at low steady state levels through an autoregulatory feedback loop involving Mdm2, which promotes rapid degradation of p53. Wild type p53 has been shown to suppress tumorigenesis and is known as a tumor suppressor. Mutation or loss of wild type p53 has been implicated in a variety of cancers, including those of the oral cavity, lung, breast, skin, and colorectal region⁷.

Human p53 is a 393 amino acid protein with three distinct functional domains: an acidic N-terminal transactivation domain, a central DNA-binding domain, and a basic C-terminal oligomerization domain. The acidic N-terminal domain is critical for p53's function as a transcriptional activator and is often a site of regulation, for example by Mdm2⁷. The basic C-terminal domain allows for tetramerization for functionality⁸. The

central DNA-binding domain (residues 100-293; Figure 2) is required for sequence specific DNA binding, which binds to 200-300 consensus sites in the human genome. The majority of p53 mutations in human tumors occur in the central DNA binding domain and affect p53's ability to bind to the wild type p53 consensus DNA sequence⁷. Most p53 mutations are single base substitutions occurring within this DNA binding domain⁹.

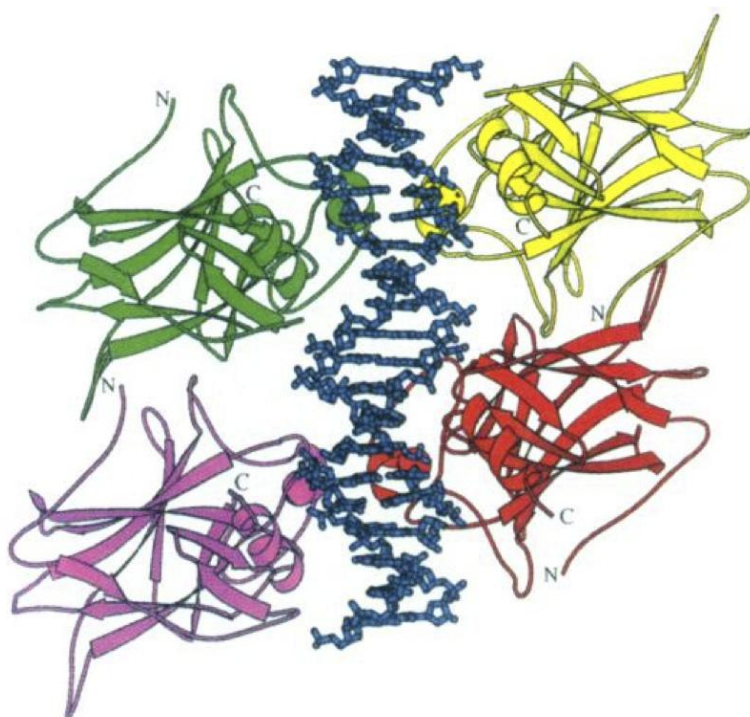


Figure 2: Model of the p53 Core Domain Tetramer DNA Complex. Core DNA binding domain of each p53 monomer interacts with DNA through its central DNA Binding domain residues 100-293¹⁰.

1.4 Mutant p53

The TP53 gene is the most commonly mutated gene in HNSCC¹¹, occurring in over 50% of HNSCC patients. TP53 mutational analysis has been used as a prognosis indicator². There are three major classes of mutant p53. The first, loss of function, results

in a non functional p53 protein. The second, dominant negative, results in a monomer that is able to tetramerize with the wild type and mask wild type function. The third, gain of function, leads to p53 protein that not only loses the wild type tumor suppressor function, but gains new oncogenic functions¹².

Mutations occurring in the DNA binding domain have been correlated with poor prognosis in HNSCC including accelerated tumor progression and decreased patient survival². Previous studies have shown high levels of mutant p53 with single point mutations in cell lines derived from HNSCC tumors¹³. These mutations occurring in the DNA binding domain lead not only to the loss of normal tumor suppressor function by interfering with p53's ability to induce apoptosis, but also the gain of oncogenic functions leading to increased proliferative and metastatic potential. Oncogenic functions gained include greater protein stability, immortalization and loss of apoptosis induced by growth factor removal¹⁴. These gain of function (GOF) mutant p53 proteins have been shown to transform immortalized fibroblast NIH/3T3 cells into malignant phenotypes and even overcome the effects of wild type p53 if present⁸.

Mutant p53 heteromerizes with wild type p53 when present and drives the formation of complexes that function aberrantly, masking wild type function. There is a large spectrum of p53 mutations with different effects at varying strengths, partially based on location of the mutation. These widespread effects of mutant p53 are due to the ability of p53 to bind to many consensus sequences within the genome¹⁴. Previous studies have shown cells expressing GOF mutant p53 have transcriptomes different from those expressing wild type p53 or p53 null cells¹⁵. Mutant p53-containing cells have also shown

elevated expression levels of genes whose protein products are critical in the regulation of cell cycle, transcription, cell matrix interactions, and DNA replication and repair, which can lead to enhanced cancer development, progression, and chemoresistance¹⁶.

1.5 p63

p63 is a member of a family of transcription factors that includes p53 and p73. p63 plays a role in stratification of epithelium during development, including the oral mucosa and the epidermis, and in cancer during adulthood. Inactivation of p63 is lethal in mice due to the lack of stratified squamous epithelia and its derivatives including appendages and salivary glands^{17,18}. p63 is rarely mutated in cancer and its expression has been found to be retained or amplified in HNSCC. Amplified expression of p63 protein can lead to an altered ratio of its two isoforms (see below). This ratio, but not necessarily the overall level of p63, has been suggested to determine biological outcome.¹⁷

p63 has been found to be expressed in the basement membrane of stratified epithelial tissues and highly expressed in some cancers deriving from these tissues, including HNSCC¹⁸. p63 has two distinct isoforms, TAp63 and Δ Np63, which are encoded by the same gene (TP63). These two isoforms have different promoters and alternative splicing patterns at the C terminus, leading to three isoforms (α , β , and γ) each for both TA and Δ Np63 of differing molecular weights (Figure 3). Distinct roles and molecular mechanisms of p63 isoforms are still controversial. TAp63 functions in a transactivating manner and has been found to contribute to maintenance of dermal and epidermal precursors, genomic stability, and lifespan¹⁷. Δ Np63 functions in a dominant

negative fashion as a transcriptional repressor¹⁸. During development, Δ Np63 plays a critical role in stratification of the epithelium. In adulthood, Δ Np63 is required for maintenance of “stemness” of stem cells within the stratified epithelia,¹⁷ which are required for normal tissue renewal and regeneration after damage¹⁹. Δ Np63 α has been found to be most prominent in the basal layers of the epithelium and the predominant isoform of p63 in HNSCC²⁰. Δ Np63 α has been found to mediate silencing of its own promoter in response to genomic stress. This decreased transcription was found to lead to altered expression patterns of p53 target genes²¹.

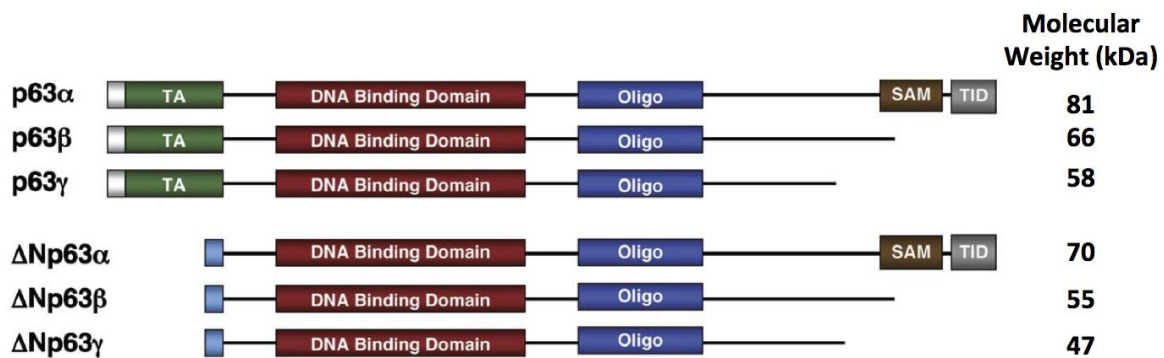


Figure 3: Isoforms of p63. Both (TA)p63 and Δ Np63 are transcribed from the TP63 gene from different promoters and undergo alternate splicing at the C terminal leading to six total isoforms, which vary by molecular weight. Modified from Barbieri, et.al. 2006¹⁸

Previous studies suggest that p63 may be binding to approximately 5800 promoters in the human genome, affecting 7% of the coding genes¹⁷. Like other members of the p53 family, active p63 functions as a tetramer¹⁷ and binds to distinct p63 consensus DNA binding sites¹⁸. p63 has the ability to tetramerize amongst isoforms and with p53 and p73.¹⁷ p63 can also bind to p53 consensus DNA binding sites in vitro and in vivo due to its high sequence and structural similarity to p53.¹⁸

In contrast to p53, p63 is not a classical tumor suppressor, but functions primarily in embryonic development. Mutant p53 has been found to interact physically with p63 resulting in loss of p63 function. In non small cell lung cancer cells, a type of epithelial cancer, mutant p53 has been seen to physically interact with TAp63 and this correlated with inhibition of p63 function²². In addition, overexpression of p63 in HNSCC is needed for the expression of over a hundred genes, many that are involved in cell motility²³.

1.6 Epidermal Growth Factor Receptor Signaling

Growth factors, such as epidermal growth factor (EGF) and transforming growth factor- β (TGF- β), play key roles in tumor progression and metastasis. EGF is a stimulator of epithelial cell growth and is capable of activating motility pathways²⁴. TGF- β regulates epithelial homeostasis and angiogenesis, thus has been found to be a stimulating factor for tumor invasion and metastasis²⁵. Previous studies have shown these growth factors affect gene expression in primary and metastatic HNSCC cells differently and could contribute to invasive properties of metastatic cells³.

The epidermal growth factor receptor (EGFR) is a transmembrane receptor with multiple polypeptide ligands, including growth factors such as EGF and TNF- α . Ligand binding leads to dimerization of the receptor and activation of downstream signaling pathways leading to altered gene expression, cellular proliferation, angiogenesis, and inhibition of apoptosis, which contribute to the development of malignancies²⁶. Increased activation of EGFR due to high expression of ligands, or receptor overexpression, has been seen in HNSCC². Stimulation of EGFR pathways has been shown to contribute to tumor

cell metastasis. EGFR activation also stimulates vascular EGF, a protein that stimulates vasculogenesis and angiogenesis²⁶. Altered expression, stimulation, and regulation of EGFR has been implicated in tumor cell proliferation and motility²⁷.

1.7 Epidermal Growth Factor Receptor Pathway Substrate 8 (EPS8)

EGFR pathway substrate 8 (EPS8) is a downstream mediator of EGFR. EPS8 has been found to be an oncoprotein linked to tumor formation. In nude mice xenografts, attenuation of EPS8 has been found to lead to a reduction in tumor formation and overexpression has been found to promote tumor growth²⁸. Altered expression of EPS8 has been found in a variety of human cancers. In colon cancer, a positive correlation has been found between EPS8 overexpression and mitogenesis²⁸. In oral squamous cell carcinoma, the EPS8 gene was found to be overexpressed and upregulation correlated with lymph node metastasis²⁹. In HNSCC, decreased levels of EPS8 have been found to impair tumorigenicity²⁷.

EPS8 has two isoforms, p97 and p68. The p97 isoform is well characterized and is the only isoform detected in human cancer cells. Structurally, EPS8 has a distinct PH domain, SH3 domain, and a degenerate SH2 domain allowing it to interact with a variety of molecules bearing the same domains. EPS8 has been found to bind directly to EGFR through a basic amino acid rich domain interacting with multiple glutamic acid residues on EGFR. Overexpression of EPS8 has been shown to enhance EGF-dependent mitogenesis, but the underlying mechanism is still unresolved.³⁰ EPS8 expression in HNSCC cells parallels expression of matrix metalloprotease 9 (MMP-9), which breaks down

extracellular matrix, playing a role in tumor cell invasion, and processes growth factors and cytokines²⁷. EPS8 has been found to deregulate the FOXM1 transcription factor leading to elevated expression of CXC-chemokines.³¹

1.8 Chemokines and Chemokine Receptors

Chemokines are small, secreted polypeptides that function as chemoattractants in the immune response. They share a conserved secondary structure and common “Greek key” supersecondary structure consisting of three antiparallel strands and a fourth β -sheet region connected by loops. Chemokines are further divided into four subfamilies based on disulfide bridge spacing. These subfamilies include the CXC subfamily (Figure 4), the CC subfamily, the C (or XC) subfamily, and the CX₃C subfamily. Chemokines serve as ligands and bear an “L” suffix. Functionally, chemokines are classified as either “homeostatic,” which are constitutively expressed and function in tissue maintenance, or “inflammatory,” which are inducible and involved in recruitment and/or maturation of pro-inflammatory cells.³²

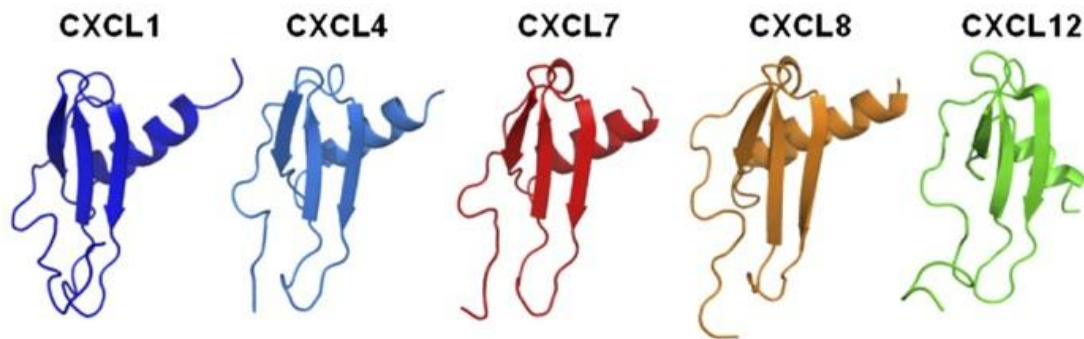


Figure 4: Structure of Members of the CXC Family of Chemokines. Members of this family of chemokines share a distinct alpha helix and beta sheet region and function as ligands.³³

Chemokines elicit their response by binding to chemokine receptors. Chemokine receptors belong to the rhodopsin-like class of G-protein-coupled 7-transmembrane (7TM) helical domain superfamily. Ligand-receptor interaction occurs at the 7TM domain, and receptor's N-terminal extracellular tail and extracellular loops also play a role. Intracellular signaling occurs through coupling of the receptor to downstream signaling cascades³².

Chemokine systems have been found to be altered in cancer. Many cancer cells have been found to overexpress chemokine receptors and metastatic sites overexpress chemokines. Due to their role in chemotaxis, chemokines are involved in initiating movement of cancer cells. Chemokines are also involved in autocrine and paracrine loops that sustain or promote cancer, and are involved in promoting angiogenesis and metastasis³².

1.9 CXCL5

CXCL5 (ENA-78 by older nomenclature) is an epithelial cell-derived neutrophil-activating peptide that binds to the CXCR2 receptor and is processed by MMP-9 after secretion. CXCL5 has been shown to play a role in a variety of cancers including a positive correlation in late stages of gastric cancer³⁴. CXCL5 also has been found to function as an angiogenic factor in non-small cell lung cancer³⁵. CXCL5 expression has been found to be altered in metastatic cells in HNSCC³⁶.

CXCL5 production has been found to contribute to enhanced invasion and proliferation in HNSCC³⁷ and is also elevated in lung cancer samples containing GOF mutant p53, leading to enhanced cell motility³⁸. Unpublished data from our lab suggest that p63 might be a negative regulator of CXCL5 expression and that mutant p53 could enhance CXCL5 expression by inhibiting p63 function. Additionally, EGF treatment has been found to lead to elevated CXCL5 expression in HNSCC cells³. Details about the relationship between GOF mutant p53, p63, EPS8 and CXCL5, and in particular the effects on EPS8 and CXCL5 promoter activity, are still unknown.

1.10 Model System

For our model system, we used a panel of cell lines isolated from patients with HNSCC. HN4 cells were isolated from a primary tongue tumor and express high levels of p63 and low levels of EPS8 and CXCL5. HN12 cells were isolated from a lymph node from the same patient and express low levels of p63 and high levels of EPS8 and CXCL5. HN4 and HN12 cells express a truncated p53 protein that is non-functional and can not tetramerize. HN6 cells were isolated from a primary tongue tumor from a patient with metastatic HNSCC and express a mutant form of p53 with a single amino acid substitution (H179L). HN13 cells are isolated from a patient with HNSCC and express mutant p53 with a single amino acid substitution (V173F) and an inframe deletion⁸.

1.11 Hypothesis

In this current study, our hypothesis is that GOF mutant p53 enhances expression of CXCL5 and other chemokines by deregulating chemokine promoter activity, either in an

EPS8-dependent or independent manner, a p63 –dependent or independent manner, or a combination thereof (Figure 5). We have two specific aims to test our hypothesis.

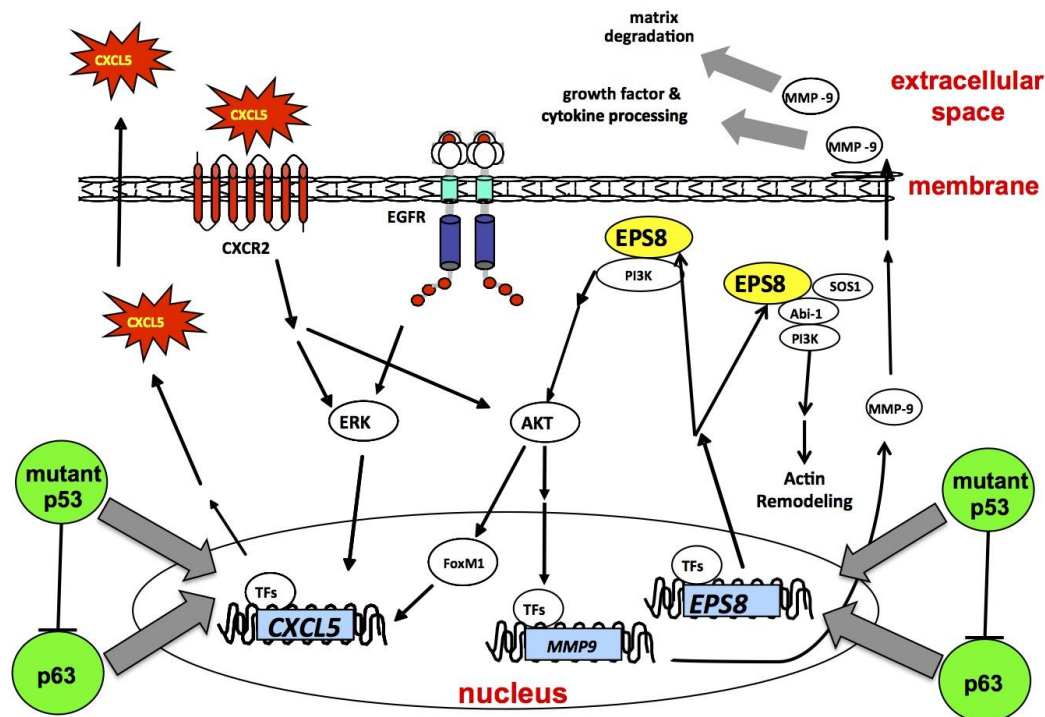


Figure 5 Hypothesis Diagram. In this study, we seek to further characterize the effect of GOF mutant p53 and p63 on EPS8 and CXCL5 expression, specifically these transcription factors effects on promoter activity.

In our first aim, we seek to determine effects of different GOF mp53 and p63 on CXCL5 (and EPS8) promoter activity and expression. Previous data from our lab suggest that GOF mp53 enhances EPS8 and CXCL5 expression and p63 inhibits EPS8 and CXCL5 expression (B.L. Field and W.A.Yeudall, unpublished).

In our second aim, we seek to determine contribution of GOF mp53 and p63 and EPS8 to migration. Previous studies have shown that GOF mp53 enhances migration of cancer cells.³⁸ While previous studies have shown that p63 inhibits cell migration in the

presence of growth factors in small cell lung carcinoma³⁹, p63's effects on migration in HNSCC have not been well characterized. EPS8 has been shown to contribute to migration in HNSCC²⁷, so we suspect if p63 is playing an inhibitory role on the EPS8 promoter and thus EPS8 expression, p63 may play an inhibitory role on migration in HNSCC.

Material and Methods

2.1 Plasmid Preparation

Plasmids containing the EPS8 promoter driving Guassia Luciferase (EPS8prGluc) or Enhanced Green Fluorescent Protein (EPS8prEGFP) and containing resistance to puromycin were obtained from GeneCopoeia (Rockville, MD) (Figure 6). A plasmid containing the CXCL5 promoter driving Firefly luciferase (CXCL5prFluc) was a generous gift from Dr A.C. Keates (Harvard Medical School). A plasmid constitutively expressing Renilla Luciferase under the control of Thymidine Kinase promoter (pRL) was obtained from Promega Corporation (Madison, WI). A plasmid containing Firefly Luciferase under the control of Herpes Simplex Thymidine Kinase promoter (pGL4.14 HSTK) was a gift from Dr. A. Waseem (Queen Mary University of London). Plasmids containing sequences encoding TAp63 (pTAp63) and Δ Np63 (pp63) were gifts from Dr. S. Deb (Dept. of Biochemistry, VCU) and Dr. Vyomesh Patel (NIDCR, Bethesda, MD). An empty vector plasmid containing Cytomegalovirus promoter, available in our lab, was used as a negative control (pCMV).

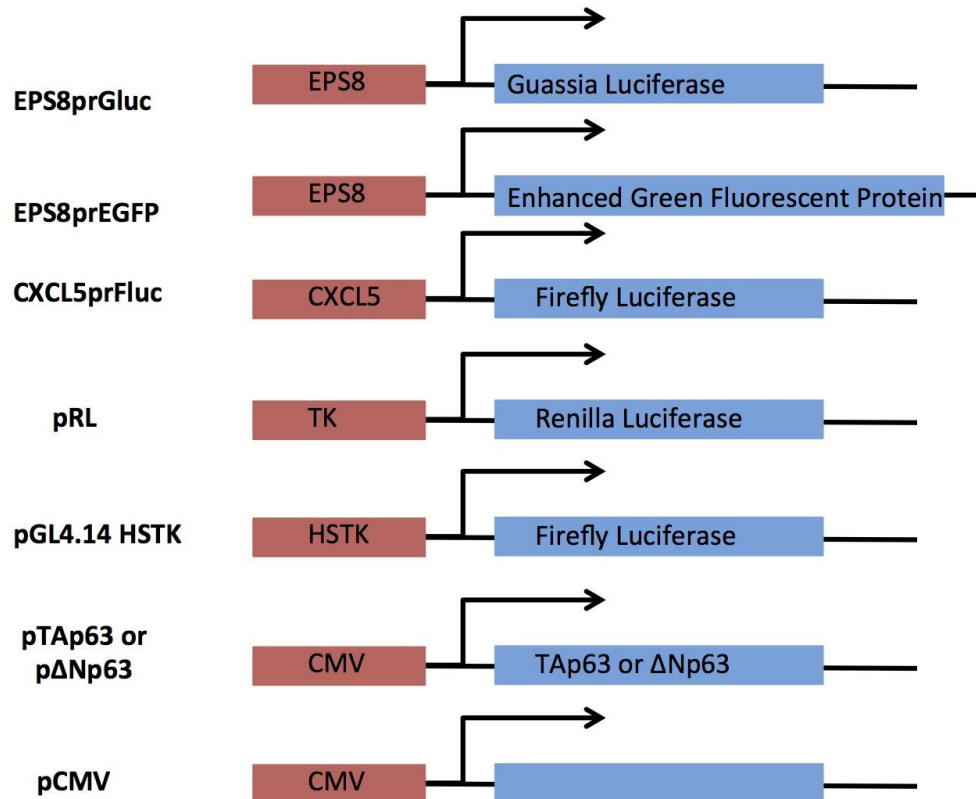


Figure 6: Plasmid Constructs. Plasmid abbreviated names and constructs utilized in our study. Promoters are shown in red. Target genes are designated in blue.

Plasmid DNA sequences were transformed into competent *E. coli* bacteria (Bioline) and grown overnight in LB broth (10 g/L typtone; 5 g/L yeast extract; 171 mM NaCl; pH 7.0) supplemented with 50μg/ml ampicillin. Plasmid DNA was prepared using the Wizard Midiprep kit (Promega; Madison, WI) according to the manufacturer's instructions. Cultures were centrifuged at 10,000 x g for 30 minutes at 4°C and the supernatant discarded. Pelleted cells were resuspended in 3 mL of Resuspension Solution (Promega; Madison, WI) by pipetting up and down in order to homogenize the sample. To the resuspended cells, 3 mL of Lysis Solution was added and inverted to mix. Then, 3 mL of Neutralization Solution (Promega; Madison, WI) was added and inverted to mix. Samples

were then centrifuged at 14,000 x g for 15 min at 4°C. The supernatant containing the DNA was filtered through gauze into a sterile 15 mL tube.

Isolated plasmid DNA was resuspended in 10 mL of resin. Mixture was drawn through a minicolumn attached to a vacuum. Once liquid had passed through, the vacuum was released. The column was washed twice with 15 mL of Column Wash Solution containing ethanol. After the second wash, the column was dried by vacuum for an additional 30 seconds after all liquid passed through. The midicolumn was separated from the reservoir and the midicolumn placed into a 1.5 mL microcentrifuge tube. The tubes were centrifuged at 10000 x g for 2 minutes. The midicolumn was then placed in a new 1.5 mL microcentrifuge tube. To the midicolumn, 300 µL of reheated 70°C water was added and then centrifuged at 10000 x g for 2 minutes. The midicolumn was discarded and eluted DNA was centrifuged for an additional 5 minutes at 10000 x g. Supernatant containing DNA was then transferred to a new microcentrifuge tube and stored at 4°C.

2.2 siRNA

Luciferase, p53, and p63 were obtained from Sigma-Aldrich, Inc. (St. Louis, MO) and are detailed in Table 1 (below). Mission siRNA Universal Negative Control (catMission siRNA Universal Negative Control (cat. # SIC001) was also purchased from Sigma Aldrich (St. Louis, MO). Dried oligonucleotides were resuspended in sterile, RNase-free water to a final concentration of 100mM.

Target Gene	siRNA Sequence
Luciferase	5'-CAUGUCAUGUGUCACAUCUC[dT][dT]-3'
p53	5'-AUGGGCCUCCGGUUCAUGC[dT][dT]-3'
p63	5'-AACAGCCAUGCCCAGUAUGUA[dT][dT]-3'

Table 1: Target Sequences for siRNA.

2.3 Cell Culture

HNSCC cells, including HN4, HN6, HN12, and HN13, were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 100 units/mL penicillin/100 µg/mL streptomycin (Invitrogen; Carlsbad, CA), 1mM sodium pyruvate (Mediatech, Inc.; Manassas, VA) and 10% fetal calf serum (ThermoScientific; Asheville, NC). Cells were incubated at 37°C in a humidified atmosphere containing 10% CO₂ and the medium was changed every 2-3 days. Cells containing the EPS8prGluc and EPS8prEGFP plasmids were maintained in medium supplemented with 1µg/mL puromycin (Enzo Life Sciences, Inc.; Farmingdale, NY)

Before cells could grow to confluency, the medium was aspirated off the plates and the cells washed thrice in sterile phosphate buffered saline (PBS, 37°C) solution to remove any remaining media. In order to detach cells, 1 mL of 0.1% trypsin (Mediatech Inc.; Manassas, VA) was added per 10cm plate and incubated for 10-15 minutes at 37°C. Detached cells were resuspended into a total volume of 10 mL of media. Cells were typically replated at a 1:10 dilution in order to maintain a culture. Cell stocks were made by transferring resuspended cells into a 15 mL tube and centrifuging at 800 RPM for 5 min

at 4°C. Remaining media was aspirated and cells were resuspended in 1 mL of 4°C Bambanker (Wako Chemicals USA; Richmond, VA). Resuspended cells were then split between two cryovials and stored at -80°C.

2.4 Cell Transfections

Cells were transfected with target DNA or siRNA by nucleofection using Mirus Ingenio electroporation solution (Mirus Bio LLC; Madison, WI). For each transfection, at least 1×10^6 cells were used as determined using a Cellometer automated cell counter and Cellometer Auto T4 software (Nexcelcom Bioscience, LLC.; Lawrence, MA). Cells were centrifuged at 800 RPM for 5 minutes at 4°C and the media aspirated off. Cells were then resuspended in 100 µL of electroporation solution and 1 µg of each DNA plasmid and/or 1 µL of 100mM siRNA was added. This solution was then transferred into a 0.2mm cuvette and placed into a Nucleofector II machine (Lonza; Allendale, NJ). The T-20 protocol was used for most transfections; T-007 protocol (high viability) was used when transfecting HN4 cells. Transfected cells were then placed into a 10cm plate of 12 mL pre-warmed DMEM. Stable cell lines containing the EPS8prGluc were selected for using DMEM supplemented with puromycin (see Cell Culture). Transiently transfected cells were assayed 2-7 days after the initial transfection.

2.5 RNA Isolation

Medium was aspirated from subconfluent cells grown in six well plates and 1 mL of TRIzol (Invitrogen; Carlsbad, CA) was added to each well. Plates were then incubated

for 5 minutes on a shaker in order to homogenize samples. Samples were then transferred into sterile 1.5 mL microcentrifuge tubes and 0.2 mL of chloroform added. Tubes were shaken vigorously for 15 seconds and then incubated at room temperature for 3 minutes. Tubes were then centrifuged for 15 minutes at 12,000 x g at 4°C in order to separate the phenol phase from the RNA-containing aqueous phase. The aqueous phases were then transferred to a sterile microcentrifuge tubes. RNA was precipitated from the aqueous phase by incubating with 0.5 mL of 100% isopropanol for 10 minutes and then centrifuged for 10 minutes at 12,000 x g at 4°C in order to pellet the RNA. The supernatant was then removed and the RNA pellets were washed with 1 mL of 75% ethanol. Tubes were then centrifuged for 5 minutes at 7,500 x g at 4°C and the wash discarded. The pellets were allowed to air dry in an inverted tube for 10 minutes at room temperature. RNA was then resuspended in 30 µL of RNase free water and incubated for 15 minutes in a 60°C water bath. Isolated RNA was then stored at -20°C. RNA concentration was determined using a spectrophotometer (Nanodrop; ThermoScientific; Asheville, NC) and ND1000 software.

2.6 Reverse Transcription

Into a sterile 1.5 mL microcentrifuge tube, 3-5 µg of isolated RNA, 1 µL of oligo(dT)₁₈ (0.5µg/µL) (SigmaAldrich, Inc.; St. Louis, MO) and dNTP mix (10mM each) (Bioline; Taunton, MA) were added and the volume was brought up to 13 µL using RNase free water. Tubes were heated at 65°C for 5 minutes, and then quickly chilled on ice before centrifugation for 30 seconds. To each tube, 4 µL of 5x First-Strand Buffer (Invitrogen; Carlsbad, CA) and 2 µL 0.1M DTT (Invitrogen; Carlsbad, CA) were added

and mixed gently by pipetting. Tubes were incubated in a 42°C water bath for 2 minutes. To each tube, 1 µL of MultiScribe reverse transcriptase (50U/µL) (Applied Biosystems; Carlsbad, CA) was added and tubes were incubated for 50 minutes in a 42°C water bath. To inactivate the reaction, tubes were heated to 70°C for 15 minutes and then stored at -20°C.

2.7 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

RNA was isolated, quantified, and reverse transcribed as described above. For each primer, a master mix was made up of 5 µL SYBR Green, 3 µL of RNase free water, and 1 µL target primer per well. To each well of a fast optical 96-well reaction plate (0.1 mL) (Applied Biosystems; Carlsbad, CA), 9 µL of master mix was added and 1 µL of target DNA was added in triplicate. As a negative control, 1 µL of water was used as a template. To construct a standard curve for each specific gene target, 1 µL of a serial dilution of previous PCR products for each primer set was utilized. The 96 well plate was sealed with an RNase-free optical adhesive film (Applied Biosystems; Carlsbad, CA), and then centrifuged for 1 minute at 800 RPM. The plate was placed into a Fast Real-Time PCR System thermal cycler (Applied Biosystems; Carlsbad, CA) and incubated at 50°C for 2 minutes then 95°C for 10 minutes. Then it was subjected to 40 cycles of 15 seconds incubation at 95°C and 1 minute at 60°C. 7500 Fast System SDS software was used to control the amplification protocol and to conduct analysis of the data.

2.8 Agarose Gel Electrophoresis

For resolving DNA, a 1% gel was made using 1g of agarose powder (Bioline; Taunton, MA) added to 100mL of 1X TAE running buffer (40 mM Tris-acetate, 1 mM EDTA) The solution was microwaved at 50% power at short intervals and mixed in between to avoid overheating. Into the completely dissolved solution, 0.5 μ L of ethidium bromide solution (10 μ g/ μ L) was added and then the solution poured onto a cooled gel tray with well comb.

DNA samples were prepared using 10 μ L of PCR product cDNA and 2.5 μ L of 5x loading buffer (0.25% bromophenol blue, 15% Ficoll 400). Samples were loaded into individual wells and Hyperladder I or II molecular weight markers (BioLine; Taunton, MA) was utilized based on size of the DNA fragments to be analyzed. Gels were electrophoresed horizontally for 30-45 minutes at a constant 100V. Gels were visualized using a UV transilluminator at 302nm and imaged using a CCD camera and software (Alpha Imager, Alpha InnoTec; Germany).

2.9 Protein Isolation

Lysis buffer was supplemented with protease inhibitors by adding 5 μ L 10mg/mL aprotinin, 5 μ L 10mg/mL leupeptin, and 5 μ L 0.1M PMSF into 1 mL of lysis buffer (20 mM HEPES, pH 7.5; 10 mM EGTA, pH 8.0; 40 mM of β -glycerophosphate; 5mL 1% NP-40 lysis buffer; and 1.50 mM MgCl₂). Medium was aspirated from six well plates of subconfluent cells and then washed twice in ice cold PBS. To each well, 100 μ L of lysis

buffer was added and placed on ice for 10 minutes. Plates were then scraped and the suspension was transferred into sterile microcentrifuge tubes. Tubes were centrifuged for 10 minutes at 10,000 RPM and 4°C. The supernatants of clarified protein lysates were then transferred into fresh microcentrifuge tubes and stored at -20°C.

To determine protein concentration, Bradford assay using BioRad Protein Assay Solution (Hercules, CA) was conducted on each sample to determine absorbance at 600nm. Concentration was then determined using a standard curve fitted for an absorbance vs. concentration plot of 50µg/mL, 25µg/mL, 10µg/mL, 5µg/mL, and 1µg/mL samples of bovine serum albumin (BSA).

2.10 Polyacrylamide Gel Electrophoresis

For a 1.5 mm thick mini-gel, a Tris-glycine SDS-polyacrylamide gel (10% acrylamide; 0.375 M Tris, pH 8.8; 0.01% sodium dodecyl sulfate (SDS), 0.01% ammonium persulfate (APS), 0.4% Tetramethylethylenediamine (TEMED)) was poured with a layer of water on top. After the resolving gel had set, the water layer was removed and a stacking gel (5% acrylamide mix; 0.125 M Tris, pH 6.8; 0.01% SDS, 0.01% APS, 1% TEMED) was poured on top and a comb inserted.

Samples were prepared by using 28 µL of the lowest concentrated sample and an equivalent microgram amount of all other samples. Samples were made up to 28 µL using lysis buffer and then 7 µL of 5x SDS loading buffer was added. Samples were heated at 95°C for 10 minutes and then loaded into the gel. Molecular weight controls consisted of 3 µL of MagicMark XP Western Standard (Invitrogen; Carlsbad, CA), and 3µL of

BenchMark Prestained Protein Ladder (Invitrogen; Carlsbad, CA). Gels were electrophoresed at 100 V over 1.5 – 2 hrs in 1X SDS-PAGE running buffer (20mM Tris-glycine pH 8.3, 2% (w/v) SDS).

2.11 Western Blot Analysis

A PVDF transfer membrane (Immobilon-P; Millipore Corp.; Bedford, MA) membrane was soaked in methanol and then soaked with filter paper and sponges in 1X transfer buffer (20mM Tris-HCl pH 7.9, 100mM NaCl, 70mM EDTA, 10% MeOH). These components were then layered with the gel and then transferred at 10 mA constant current overnight in 1x transfer buffer.

After overnight transfer, the membrane was dehydrated in methanol and then allowed to completely air dry on filter paper. The dried membrane was then re-wet in methanol and then washed for 10 minutes in TBS (10 mM Tris HCl pH 7.6, 150 mM NaCl) supplemented with 0.5% Tween 20 (Fisher Bioreagents; Rockville, MD) (T-TBS). The membrane was then removed and blocked in 5% milk in 1x T-TBS for at least 1 hour on a shaker at room temperature or overnight at 4°C. The membrane was then removed and incubated in primary antibody on a shaker for 1 hour at room temperature or overnight at 4°C. The membrane was then washed three times at ambient temperature for 10 minutes in TTBS. The membrane was then incubated in animal specific secondary antibody on a shaker for 1 hour at ambient temperature. Anti-goat secondary was obtained from MP Biomedical. Anti-mouse was obtained from Cell Signalling. The membrane was then washed three times for 10 minutes or six times for 5 minutes in TTBS.

Primary Antibody	Raised Against	Dilution	Secondary Antibody (Dilution)
actin	C-terminus	(1:1000)	Anti-goat (1:10,000)
EPS8	aa 628-821	(1:5000)	Anti-mouse (1:10,000)
p53	aa 11-25	(1:1000)	Anti-mouse (1:10,000)
p63	aa 1-205 (ΔN N-terminus)	(1:250)	Anti-mouse (1:2,000)

Table 2: Actin (cat. # IL-6), p53 (cat. # D01), and p63 (cat. # 4A4) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). EPS8 antibody (cat. # 610144) was obtained from BD Biosciences (San Jose, CA).

The membrane was developed with 500 μ L each of Western Lightning Oxidizing Reagent Plus and Western Lightning Enhanced Luminol Reagent Plus (PerkinElmer Life Sciences Inc.; Billerica, MA). The developing solution was pooled on Saran wrap and the membrane placed face down for 3 minutes. Excess developing solution was squeezed off and the membrane imaged using Blue X-ray film (Phenix Research Products; Candler, NC) with a Kodak X-OMAT 2000 Processor.

2.12 Luciferase Assay

Medium was aspirated from a six well plate and wells washed 1x in PBS and aspirated. 500 μ L of 1x Passive Lysis Buffer, made from the provided 5x solution (Promega; Madison, WI) diluted in sterile water, was added to each well. The plate was placed on a shaker for 15 minutes at ambient temperature and then cell lysate and debris were scraped into sterile 1.5 mL microcentrifuge tubes. Tubes were centrifuged for 30 seconds and the cleared supernatant transferred into sterile microcentrifuge tubes. Stop

and Glo working solution was made for 100 μ L per sample by diluting 50x Stop & Glow reagent in Stop & Glow Buffer (Promega; Madison, WI).

In a tube of 100 μ L of Luciferase Assay Reagent II (LAR II), 20 μ L of sample was added and mixed by pipetting up and down. The sample was then placed into a Glomax 20/20 luminometer (Promega; Madison, WI) where a protocol with 10 second integration was run. After the first reading, 100 μ L of Stop and Glo reagent was added and vortexed for 15 seconds at 3000 RPM. The sample was then placed back into the luminometer for a second reading. The individual readings recorded in relative light units (RLU) and ratio of the first reading to the second reading .

Luminometer readings are dependent on luciferase enzymes expressed, which vary based on the plasmid construct utilized (Figure 6). Firefly luciferase (FLuc) reacts with LAR II and emits within the first reading. The Stop and Glo reagent quenches this reaction and also serves as a substrate for Gaussia luciferase (Gluc) and Renilla luciferase (pRL), which both emit within the second reading. Unless a stable cell line is used, these reporter plasmids were cotransfected with a normalizing plasmid in order to account for transfection efficiency (Table 3). These normalizing plasmids express constitutively expressed luciferase enzymes that react and emit differently than the plasmid containing the promoter of interest.

Promoter of Interest Plasmid	Cotransfected Plasmid	Reading 1 (RLU1)	Reading 2 (RLU2)
CXCL5prFluc	pRL	CXCL5pr activity (Fluc)	Transfection efficiency (pRL)
EPS8prGluc	pGL4.14 HSTK	Transfection efficiency (pGL4.14 HSTK)	EPS8pr activity (Gluc)
	None (stable cell line)	background	EPS8pr activity (Gluc)

Table 3: Luciferase Assay. Depending on promoter construct used, promoter of interest activity will be reported at different reading based on substrates for enzymes utilized.

2.13 Statistical Analysis

Data were analyzed using GraphPad QuickCalcs software

(<http://www.graphpad.com/quickcalcs>) to conduct unpaired t-test to compare two means.

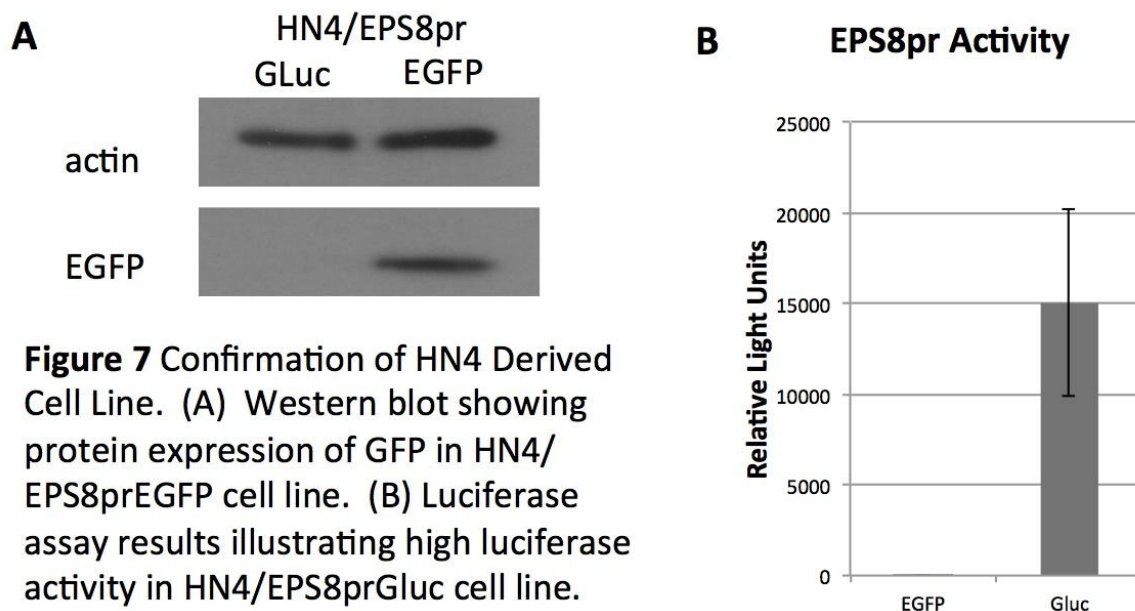
A p value of less than 0.05 was considered statistically significant for this study.

Results

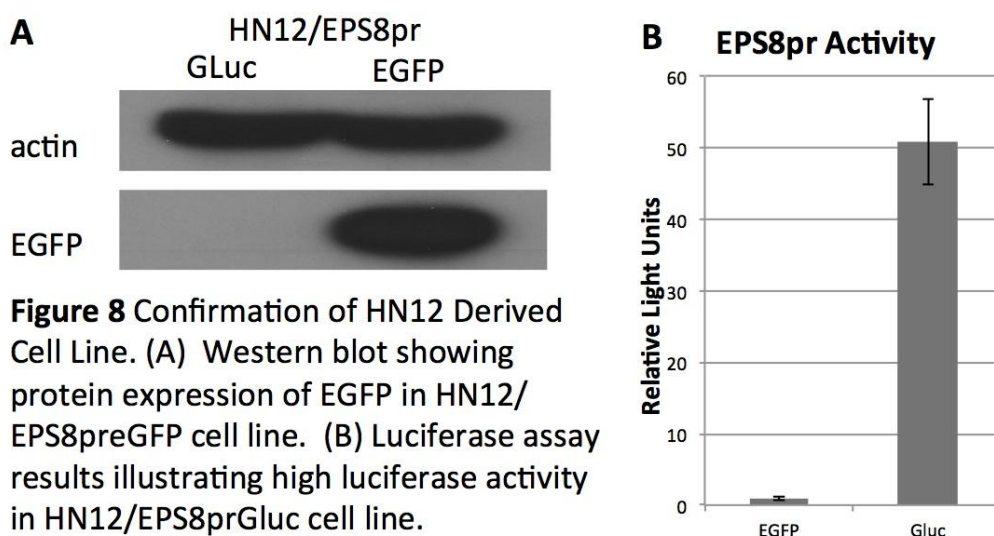
3.1 Creation of stable cell lines

In order to study EPS8 promoter activity more easily, stable cell lines were created by transfecting a plasmid containing the EPS8 promoter driving expression of either Guassia Luciferase (EPS8prGluc) or Enhanced Green Fluorescent Protein (EPS8prEGFP), in addition to puromycin resistance, into HN4 and HN12 cells. Such cell lines would allow for the study of promoter activity of the plasmid construct without the need to cotransfect with another DNA plasmid for normalization. After transfection of DNA, the cells were cultured in media supplemented with puromycin for 10-15 days or until discreet colonies became visible. At the end of this period, protein and luciferase assay samples were isolated by the standard protocols (as described above).

Figure 7A shows a Western blot image of protein samples derived from the HN4/EPS8prEGFP and HN4/EPS8prGLuc cell lines, illustrating the expression of EGFP in the created HN4/EPS8prEGFP cell line, but not in cells transfected with the luciferase plasmid. Conversely, luciferase assay data from these cell lines shows high activity of the EPS8prGluc in HN4 cells as expected (Figure 7B), but not in the EGFP-transfected cells.



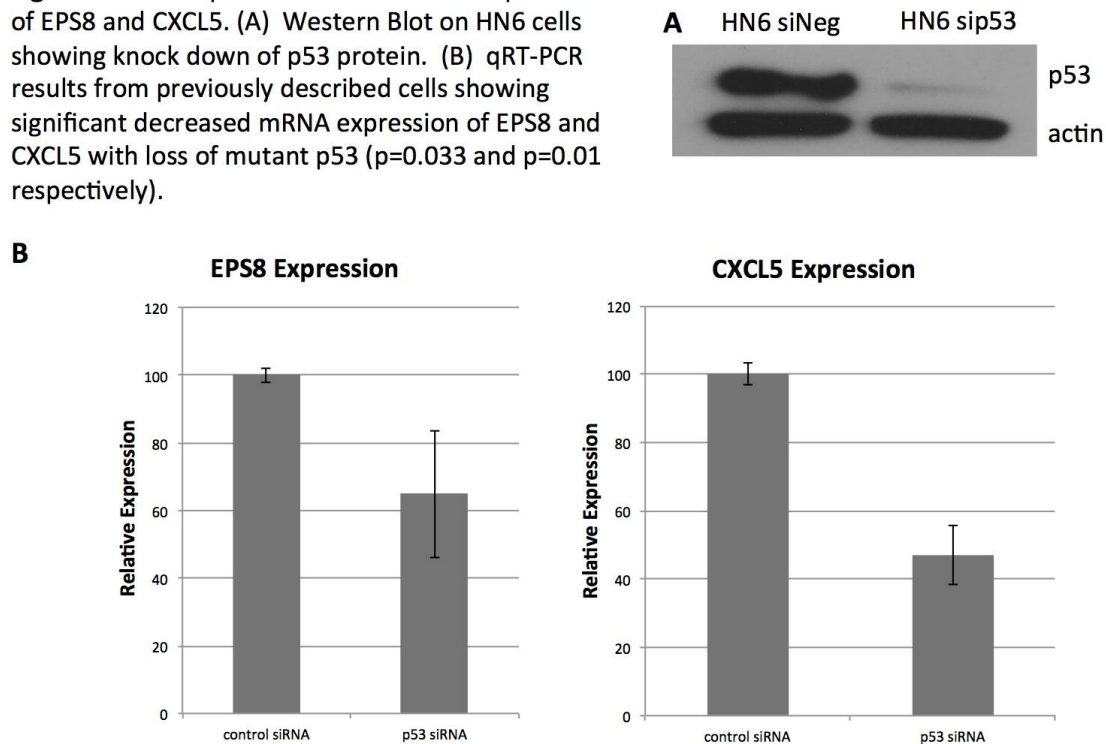
HN12 derivative cell lines also exhibited high expression of EGFP protein by Western blot (HN12/EPS8prEGFP cells, Figure 8A) and high activity of the EPS8prGluc construct by luciferase assay (Figure 8B). These findings confirm the creation of the desired stably transfected cell lines. Similar cell line derivatives were also created using HN6 cells by other members of the lab.



3.2 Mutant p53 enhances EPS8 and CXCL5 expression and enhances migration

Previous data from our lab indicated an enhancing role of mutant p53 on EPS8 and CXCL5 expression in HNSCC (B.L. Field and W.A. Yeudall, unpublished), which we sought to confirm in a different cell line in this study. As seen in Figure 9A, the knock down of mutant p53 (H179L) protein in HN6 cells leads to a significant decrease in mRNA expression of EPS8 and CXCL5 mRNA ($p=0.033$ and $p=0.01$ respectively) (Figure 9B).

Figure 9 Mutant p53 Leads to Increased Expression of EPS8 and CXCL5. (A) Western Blot on HN6 cells showing knock down of p53 protein. (B) qRT-PCR results from previously described cells showing significant decreased mRNA expression of EPS8 and CXCL5 with loss of mutant p53 ($p=0.033$ and $p=0.01$ respectively).



Mutant p53 has been shown to contribute to cellular migration³⁸, although the mechanism is unclear. Therefore, we tested to see if there was a correlation between mutant p53 knockdown, reduced EPS8, reduced CXCL5 and altered motility. As seen in Figure 10, in our study the knock down of this mutant p53 protein also leads to a

significant decrease in cellular migration as seen in wound closure assay ($p=0.0003$). This is consistent with previous data obtained in lung cancer cells¹⁶ and implicates mutant p53-dependent regulation of EPS8, a known mediator of actin reorganization, as a potential mechanism to explain the enhanced migration of cancer cells containing mutant p53.

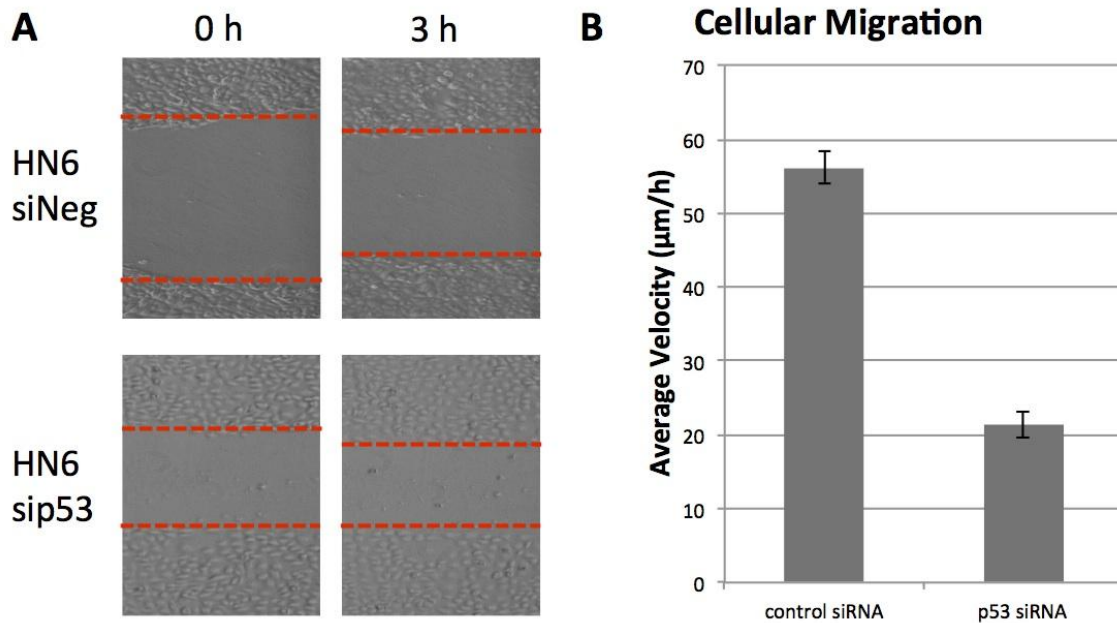


Figure 10 Mutant p53 Enhances Cellular Migration. Scratch assay results from cells described in Figure 9, original magnification x50 (A). Observed a significant decrease in migration with loss of mutant p53 ($p=0.0003$) (B). Protein levels of p53 were confirmed by Western Blot (Fig9A).

3.3 p63 inhibits EPS8 and CXCL5 expression and cellular migration

Previous data from our lab indicate an inhibitory role of p63 on CXCL5 expression (B.L. Field and W.A.Yeudall, unpublished). In Figure 11, the knock-down of p63 mRNA expression using siRNA leads to a significant increase expression of CXCL5 mRNA ($p=0.001$), consistent with previous findings. In addition, EPS8 levels increased when p63 expression was repressed by siRNA (Figure 11; $p=0.01$).

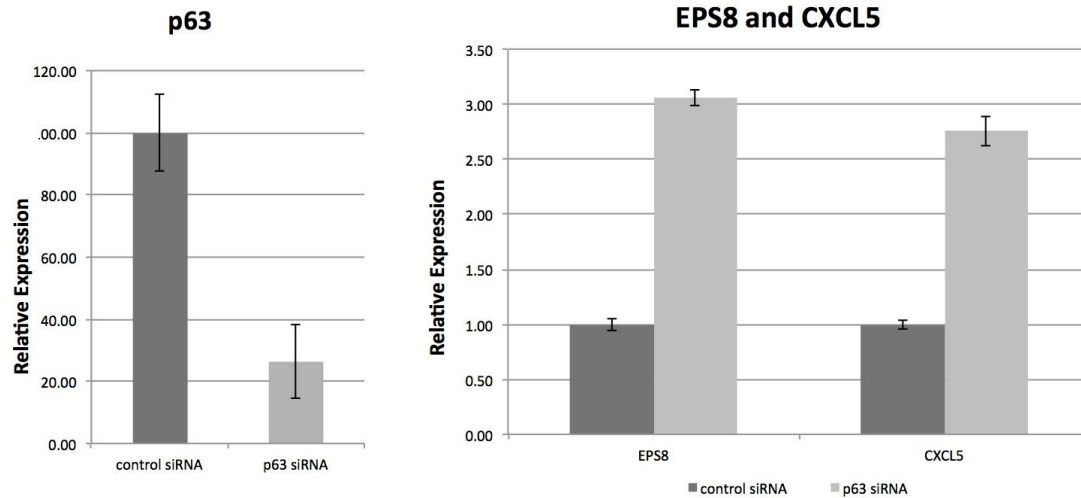


Figure 11 p63 Inhibits EPS8 and CXCL5 Expression. qRT-PCR results from HN4 cells illustrating a significant increase in EPS8 and CXCL5 mRNA expression with the knock down of p63 ($p=0.01$ and $p=0.001$ respectively).

Previous studies have provided evidence of mutant p53 inhibiting p63 function, thereby promoting cellular invasion in small cell lung cancer cells²². We sought to characterize the effect of p63 independent of mutant p53 by utilizing HN4 cells that do not express a functional p53. As seen in Figure 12, knock down of p63 in these cells using siRNA is associated with enhanced cellular migration.

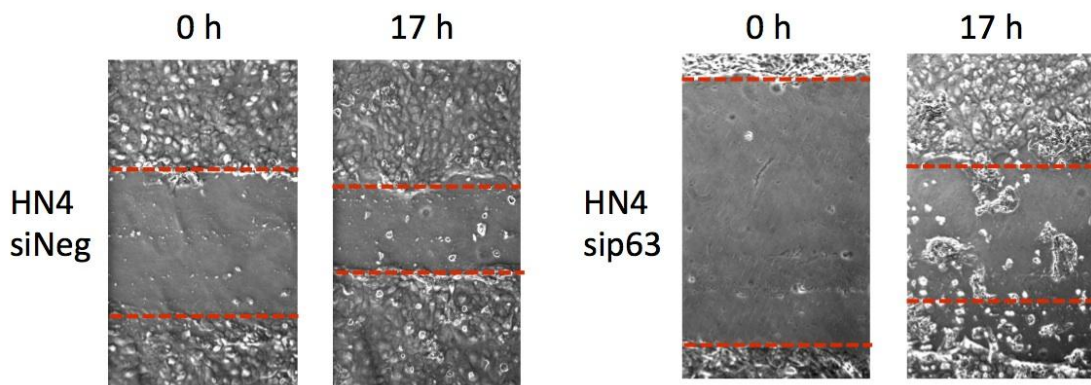


Figure 12 p63 Inhibits Cellular Migration. Scratch assay images from HN4 cells transfected with control siRNA or siRNA for p63. Increase migration is observed with the loss of p63.

3.4 Mutant p53 enhances EPS8 promoter activity

Preliminary data from our laboratory indicate that mutant p53 might enhance EPS8 expression. In this study, we further characterized this relationship in terms of promoter activity. As seen in Figure 13A, the knock down of mutant p53 protein in HN6 cells is achieved using siRNA. Isolates from the same transfection exhibited a significant decrease in EPS8 promoter activity with the loss of mutant p53 protein ($p=0.001$; Figure 13B).

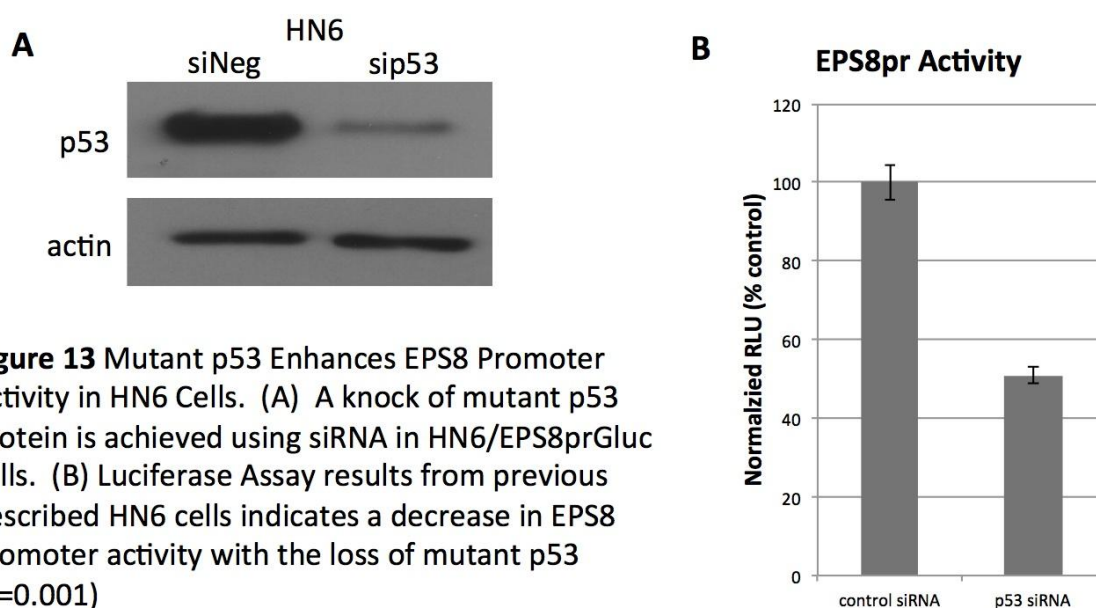


Figure 13 Mutant p53 Enhances EPS8 Promoter Activity in HN6 Cells. (A) A knock of mutant p53 protein is achieved using siRNA in HN6/EPS8prGluc cells. (B) Luciferase Assay results from previous described HN6 cells indicates a decrease in EPS8 promoter activity with the loss of mutant p53 ($p=0.001$)

In a complementary experiment, the knock down of mutant p53 protein using p53 siRNA was carried out in HN13 cells transiently transfected with the previously described EPS8prGluc promoter (Figure 14A). Isolates from this transfection exhibited a significant decrease in EPS8 promoter activity with the loss of mutant p53 (Figure 14B). This provides further evidence of mutant p53's enhancing role on EPS8 expression and identifies increased promoter activity as part of the mechanism.

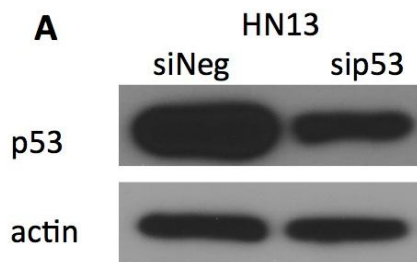
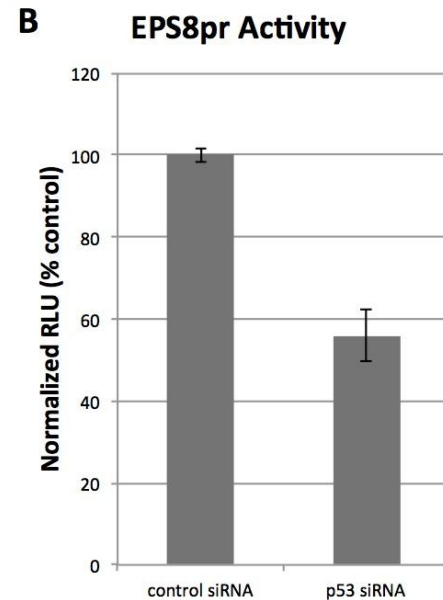
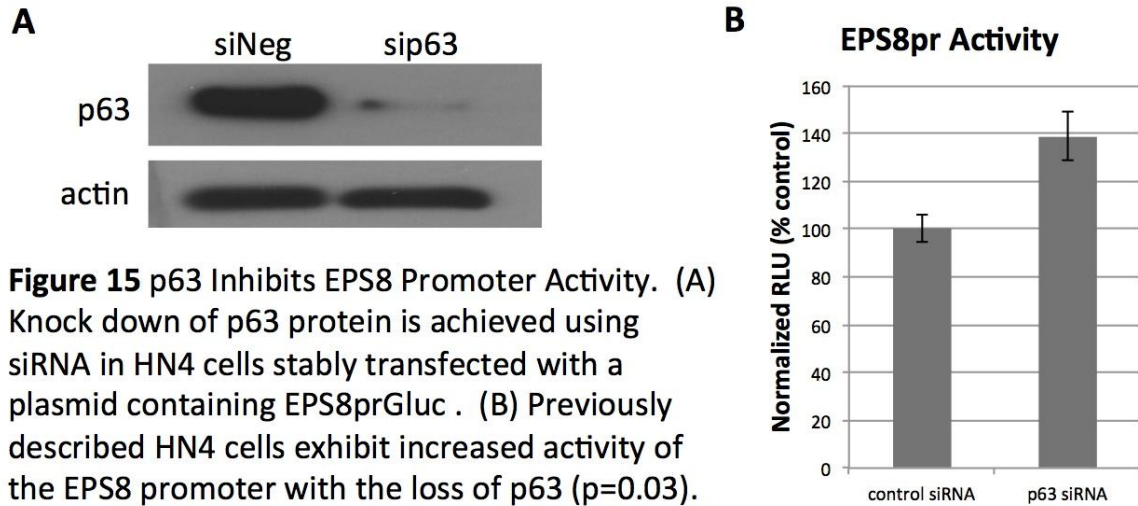


Figure 14 Mutant p53 Enhances EPS8 Promoter Activity. (C) A knock down of mutant p53 protein is seen using siRNA in HN13 cells transiently transfected with EPS8prGluc. (D) Luciferase assay results from previously described HN13 cells indicate a decrease in EPS8 promoter activity with the loss of mutant p53 ($p=0.003$).



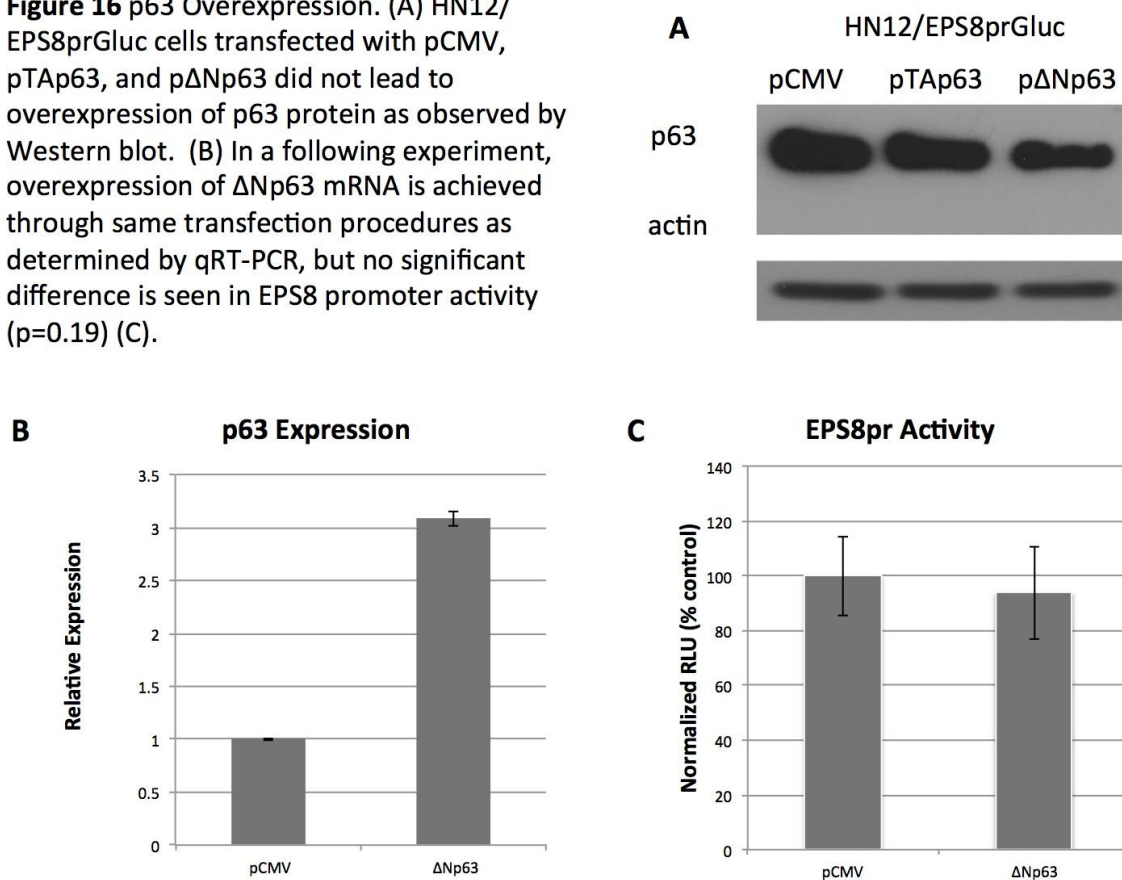
3.5 p63 inhibits EPS8 promoter activity

To explore the inhibitory role of p63 on EPS8 expression further, we utilized the stably transfected HN4/EPS8prGluc cell line created (see above). As seen in Figure 15A, p63 protein expression is reduced efficiently in these cells using siRNA. This loss of p63 was associated with an increase in EPS8 promoter activity ($p=0.03$; Fig 15B). This is consistent with our previous expression data indicating an inhibitory role of p63 on EPS8 expression.



A complementary experiment was performed in the HN12/EPS8prGluc cell by transfection a control, TAp63 and Δ Np63 plasmids in order to overexpression p63 in these cells that typically express a low level of p63. During initial studies, the overexpression of p63 protein was not observed after transfection using pTAp63 and p Δ Np63 (Figure 16A). Interestingly, p63 probed for by Western blot were all of the same molecular weight, which would not be expected if the transfection was successful. This leads us to believe that the p63 might represent Δ Np63 α , which is the predominant form in HNSCC¹⁷. In a repeated trial, overexpression of Δ Np63 mRNA is seen following the same transfection procedure (Figure 16B), but no significant difference is seen in EPS8 promoter activity (Figure 16C). This could be due to discrepancies between mRNA and protein levels of p63.

Figure 16 p63 Overexpression. (A) HN12/ EPS8prGluc cells transfected with pCMV, pTAp63, and pΔNp63 did not lead to overexpression of p63 protein as observed by Western blot. (B) In a following experiment, overexpression of ΔNp63 mRNA is achieved through same transfection procedures as determined by qRT-PCR, but no significant difference is seen in EPS8 promoter activity ($p=0.19$) (C).



3.6 Mutant p53 enhances CXCL5 promoter activity

In order to characterize mutant p53's effects on CXCL5 expression further, HN6 and HN13 cells were transiently transfected with a plasmid containing the CXCL5 promoter driving expression of Firefly luciferase (CXCL5prFluc), Renilla luciferase plasmid, and siRNA. As seen in Figure 17A, the knockdown of mutant p53 protein was achieved in HN6 cells and is associated with a decrease in CXCL5 promoter activity ($p=0.001$; Fig. 17B).

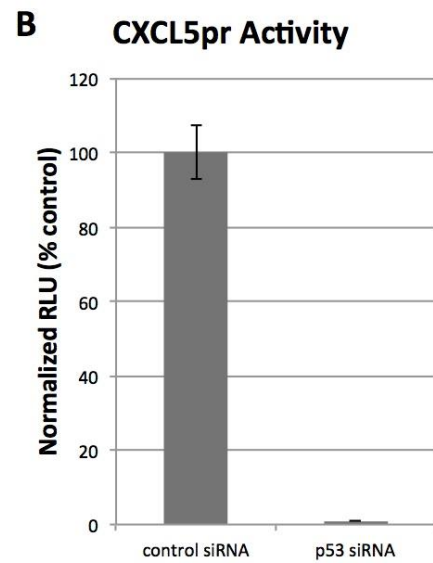
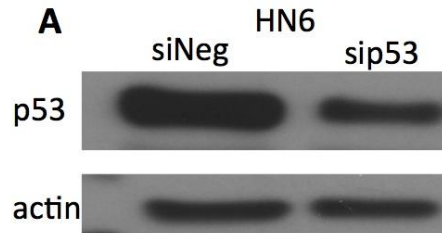


Figure 17 Mutant p53 Enhances CXCL5 Promoter Activity in HN6 Cells. (A) Knock down of mutant p53 protein is achieved using siRNA in HN6 cells cotransfected with CXCL5prFluc. (B) A decrease in CXCL5 promoter activity is seen previously described HN6 cells with the knockdown of mutant p53 protein ($p=0.001$).

In a complementary experiment, the knockdown of mutant p53 was achieved in HN13 cells (Fig 18A) and is associated with a decrease in CXCL5 promoter activity ($p=0.001$; Fig 18B). These findings are consistent with previous data from our lab indicating an enhancing role of mutant p53 on CXCL5 expression levels and extends previous work by indicating that this is due, at least in part, to increased promoter activity.

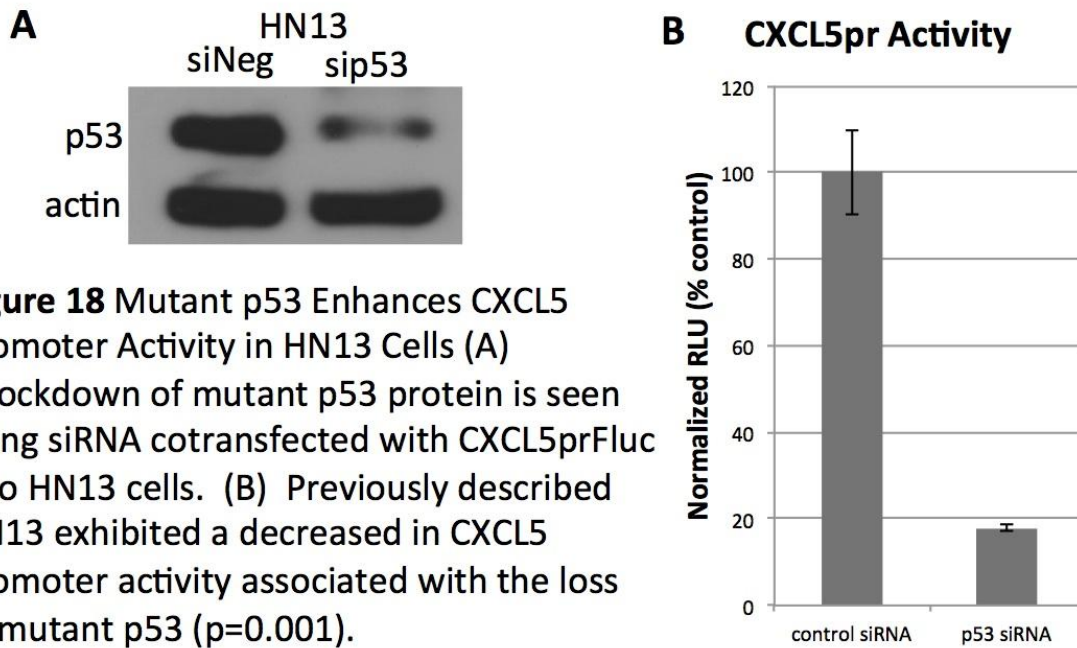


Figure 18 Mutant p53 Enhances CXCL5 Promoter Activity in HN13 Cells (A) Knockdown of mutant p53 protein is seen using siRNA cotransfected with CXCL5prFluc into HN13 cells. (B) Previously described HN13 exhibited a decreased in CXCL5 promoter activity associated with the loss of mutant p53 ($p=0.001$).

3.7 p63 inhibits CXCL5 promoter activity

Previous data from our lab has indicated an inhibitory role of p63 on CXCL5 expression. In order to further study p63's effects on CXCL5 expression, HN4 cells were transiently transfected with CXCL5prFluc plasmid, Renilla Luciferase, and siRNA in order to study CXCL5 promoter activity. As seen in Figure 19A, knockdown of p63 protein was achieved using siRNA and is associated with an increase in CXCL5 promoter activity (Figure 19B). This provides further evidence of p63's inhibitory role on CXCL5 expression and that the effects are manifest through altered promoter activity.

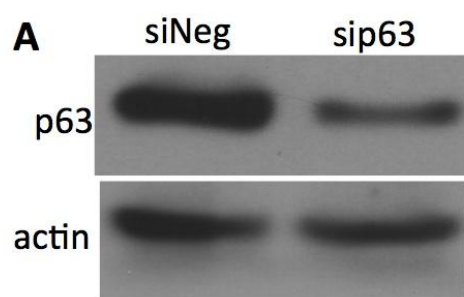
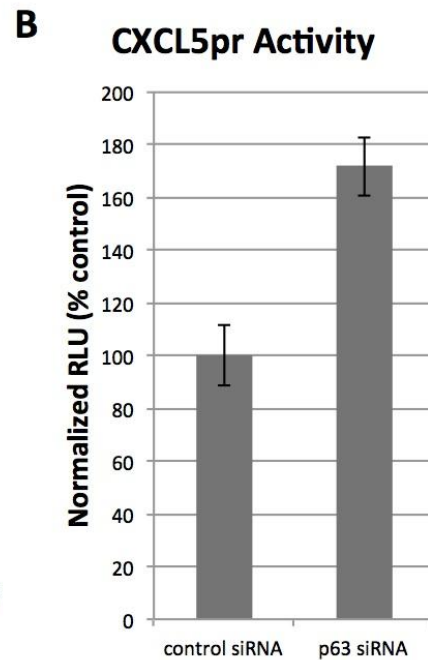


Figure 19 p63 Inhibits CXCL5 Promoter Activity. (A) Knockdown of p63 protein using siRNA is achieved by cotransfecting with CXCL5prFluc into HN4 cells. (B) Increased in CXCL5 promoter activity is associated with the loss of p63 in previously described HN4 cells



Discussion

4.1 GOF Mutant p53 Enhances HNSCC Tumorigenesis

Previous findings from our lab illustrate an enhancing role of GOF mutant p53 in cellular migration by expressing GOF mutant p53 in HN4 cells³⁸. In this current study, we used different cell lines, HN6 and HN13, isolated from HNSCC tumors that express endogenous GOF mutant p53. After delivery of siRNA, we confirmed the knockdown of p53 protein in these cell lines using Western Blot. We observed decreased migration of HN6 cells when mutant p53 was inhibited, indicating a role for GOF mutant p53 in cellular migration. These findings are consistent with previous studies in non small cell lung cancer cells, in which GOF mutant p53 has been found to contribute to cellular migration and invasion^{22,39}.

In HN6 cells, we also observed decreased EPS8 and CXCL5 mRNA levels with the loss of mutant p53. This indicates an enhancing role of GOF mutant p53 on EPS8 and CXCL5 expression, which supports previous findings from our lab³⁸ (B.L. Field and W.A.Yeudall, unpublished). Our study adds further evidence to a possible GOF mutant p53-mediated mechanism leading to the overexpression of EPS8 and CXCL5, which have been found to contribute to tumorigenesis in HNSCC^{27,37}.

The enhanced migration associated with GOF mutant p53 could be due to increased EPS8 and CXCL5 expression in these cells. In previous studies, the overexpression of EPS8 has been found to increase cellular proliferation and migration, but alone was found to have no significant effect on invasion²⁷. In our study, the effect of EPS8 and CXCL5

overexpression could both be contributing to increased migration. Increased expression of EPS8 due to the presence of GOF mutant p53 would effect actin remodeling and increase secretion of MMP-9, which contributes to extracellular matrix degradation and processing of CXCL5. Through these pathways, the increased expression of EPS8 due the presence of GOF mutant p53 could enhance HNSCC tumorigenesis. The increased expression of EPS8 could be contributing to the increased expression of CXCL5 through the upregulation of FOXM1³¹. GOF mutant p53 could also be increasing CXCL5 through an EPS8 independent pathway. The increased expression and secretion of the chemokine due to the presence of GOF mutant p53 would establish a more concentrated chemoattractive gradient than cells with GOF mutant p53 knocked down. In HNSCC tumorigenesis, this would lead to enhanced migration of endothelial cells for establishment of a tumor blood supply in tumor expressing GOF mutant p53. This proposed mechanism is in line with previous studies that have associated the down regulation of CXCL5 with inhibition of squamous carcinogenesis³⁷. In order to investigate the mechanism by which GOF mutant p53 enhances EPS8 and CXCL5 mRNA expression further, promoter activity of these genes was studied.

Wild type p53 protein functions as a transcription factor either binding directly to promoters of target genes and/or recruiting other transcription factors to target promoters⁷. In addition, GOF mutant p53 cell lines have exhibited different transcriptomes than those of wild type p53 or even other GOF mutant p53s¹⁵. Previous studies in non-small cell lung cancer have found GOF mutant p53 down regulates promoters of wild type p53 target genes^{40,41}. Thus we suspected that the mutant p53 present in our HNSCC lines may be

affecting EPS8 and CXCL5 promoter activity, leading to increased mRNA expression.

Our findings identify novel target promoters of GOF mutant p53. After confirming the knockdown of p53 protein using siRNA in HN6 and HN13 cells, we observed a significant decrease in EPS8 and CXCL5 promoter activity associated with the loss of GOF mutant p53 as determined by luciferase assay. Our findings support our hypothesis of an enhancing role of GOF mutant p53 on EPS8 and CXCL5 promoter activity and are consistent with our expression studies, thus elucidating further the mechanism in which GOF mutant p53 contributes to HNSCC tumor progression. As a transcription factor, GOF mutant p53 can be binding directly to these promoters, or recruiting other transcription factors, or a combination of both. GOF mutant p53 has been found to bind directly to DNA leading to altered promoter activity, which would be a mechanism by which GOF mutant p53 might be functioning in our study⁴². Additional experiments need to be conducted in order to further elucidate the mechanism by which GOF mutant p53 regulates EPS8 and CXCL5 promoter activity.

Our current findings support previous studies showing the deregulation of chemokine expression in a variety of cancers⁴³. In addition, both EPS8²⁹ and CXCL5³⁷ overexpression have been implicated in HNSCC. The elevated promoter activity leading to increased mRNA expression of EPS8 and CXCL5 supports the hypothesis that GOF mutant p53 proteins play a role in HNSCC tumorigenesis.

4.2 p63 plays an inhibitory role in HNSCC Tumorigenesis

Unpublished data from our lab has indicated an inhibitory role of p63 on CXCL5

expression, which may be one mechanism by which mutant p53 exerts its effects. In this study, we wanted to characterize p63's role independent of mutant p53. As a homolog of p53, p63 functions as a transcription factor that binds to consensus DNA sequences on target promoters¹⁷. Thus we sought to characterize the effects of p63 on promoter activity of two possible target genes, EPS8 and CXCL5. Previous studies from our lab have shown EGF treatment of HNSCC cells leads to high expression of CXCL5 (B.L. Field and W.A. Yeudall, unpublished), though it unknown if this effect is mediated by EPS8, which has been shown to increase CXCL5 expression through FOXM1³¹.

In order to study p63's role in HNSCC, we utilized HN4 and HN12 cells from HNSCC patients, which express high and low levels of p63, respectively. In addition, these cell lines express a truncated p53 protein, which is non functional and unable to oligomerize with p53 or p63. First, we studied p63's effects on EPS8 and CXCL5 expression. We knocked down p63 in HN4 cells using siRNA, which resulted in increased mRNA expression of EPS8 and CXCL5. Previous studies have independently associated the loss of p63¹⁸ and the overexpression of EPS8²⁷ and CXCL5³⁷ to HNSCC, but none have directly linked p63 to EPS8 and CXCL5 expression. We also observed increased cellular migration with the loss of p63. A previous study utilized cells from a hypopharyngeal tumor from a patient with HNSCC and found both the upregulation and downregulation of 127 genes due to the loss of p63. Of these affected genes, 20% were related to cell motility and p63 was found to bind directly to the promoters of a select few, indicating a role of p63 in cellular motility and invasion²³, which coincides with our findings. The inhibitory role of p63 on these target genes expression could contribute

impaired HNSCC tumorigenesis, since both EPS8 and CXCL5 overexpression has been shown in HNSCC. Based on these findings and previous studies, we believe that p63 could be exerting its effects by altering the activity of target gene promoters .

In order to study the mechanism by which p63 affects EPS8 and CXCL5 mRNA expression levels further, we studied their promoter activities by luciferase assay. In parallel experiments, we observed increased EPS8 and CXCL5 promoter activity associated with the loss of p63 protein as confirmed by Western Blot. These findings coincide with previous studies in which p63 was found to alter gene expression of a large array of genes and was found to bind directly to several promoters directly⁴². It is important to note that the p63 probed for by Western Blot was observed at an approximate molecular weight of 70 kDa, indicating $\Delta Np63\alpha$. This is consistent with previous findings that have shown this isoform to be the predominant form in HNSCC²⁰. Since these experiments were conducted in HN4 cells, p63 present must be functioning independent of p53, since the p53 present is non functional and truncated preventing oligomerization.

Our findings support those of a previous study in which $\Delta Np63\alpha$ was found to be a negative regulator of promoter activity of several gene targets of wild type p53, including p21 and 14-3-3 σ , by binding directly to their promoters⁴⁴. While previous studies from our lab have shown decreased expression of CXCL5 due to GOF mutant p53's inhibition of p63 function, our current findings indicate a new role and mechanism of action for p63 independent of mutant p53 in HNSCC (B.L. Field and W.A. Yeudall, unpublished).

Additional studies to overexpress p63 protein in HN12/EPS8prGluc cells were attempted. During initial experiments, overexpression of p63 protein was not observed by

Western blotting after transfection with plasmids containing isoforms of p63. In one trial, overexpression of p63 mRNA was achieved by the same DNA transfection procedure, but no significant effect was observed on EPS8 promoter activity, nor was an increased level of p63 protein detectable by Western blotting. These findings could be due to different p63 mRNA and protein levels after transfection during this trial. Future studies comparing mRNA and protein levels of p63 and their effects on target promoter activity should be conducted in order to resolve this question.

4.3 Limitations of Current Study

In this study, we relied on in vitro models using cell lines derived from HNSCC patients. Since cells were grown in an isolated culture dish with controlled media, the model is not entirely representative of the human body, where tumor cells have a more complex and dynamic microenvironment. Tumor cells within the body respond to a variety of signaling factors released by other cells. This is an important aspect of tumor cell progression and metastasis, but is lost when in the cell culture procedure used in our study.

Scratch assay studies were useful in studying cellular migration across a two dimensional surface, but this is not necessarily representative of cellular migration that occurs in three dimensions within the body. Other migration assays, such as transwell migration assay (see below), could provide more insight into cellular migration in HNSCC.

4.4 Future Studies

These experiments should be repeated in HNSCC cell lines derived from metastasis sites, particularly lymph nodes of the head and neck, which are the primary site of HNSCC

metastasis. Previous studies have shown primary and metastasis sites having varying gene expression profiles in HNSCC³. As transcription factors, GOF mutant p53 and p63 could be contributing to the varying transcription of genes under their regulation in different tumor sites.

To study cellular migration in a more representative model, transwell migration assays, which have been used with other cell lines derived from HNSCC cells, could be utilized³⁸. These assays would also allow the study of the effects of chemoattractants and growth factors on tumor cell migration, in particular CXCL5, which our studies have shown is overexpressed in HNSCC. In addition, migration across a 3D matrix, such as Matrigel, should be conducted in order to study migration in three dimensions.

In order to study GOF mutant p53 and p63 proteins' effects on the EPS8 and CXCL5 promoters, protein-DNA binding studies such as ChIP assays could be conducted. This assay would allow us to investigate if GOF mutant p53 and p63 are acting directly through binding to these target promoters or if they are functioning through recruitment of other transcription factors.

In addition, the role of p73 in EPS8 and CXCL5 regulation in HNSCC should also be studied. p73, like p63, can be transcribed from two different promoters leading to TAp73 and Δ Np73. Δ Np73 has been found to be the predominating form in HNSCC, while TAp73 transcript alterations have been found in HNSCC⁴⁵. Like other members of its family, p73 could be affecting gene expression by deregulation of target promoters in HNSCC.

4.5 Conclusions

In this study, we were able to identify new relationships between GOF mutant p53, p63, EPS8 and CXCL5 in HNSCC (Figure 20). We found that GOF mutant p53 enhances EPS8 and CXCL5 expression by upregulating promoter activity of these genes. We also found that p63 inhibits EPS8 and CXCL5 expression, independent of GOF mutant p53, and does so by decreasing promoter activity. By further elucidating the mechanism of action of these key molecules in HNSCC, therapies targeted at GOF mutant p53 or even CXCL5 could be developed to fight tumor development and progression in HNSCC.

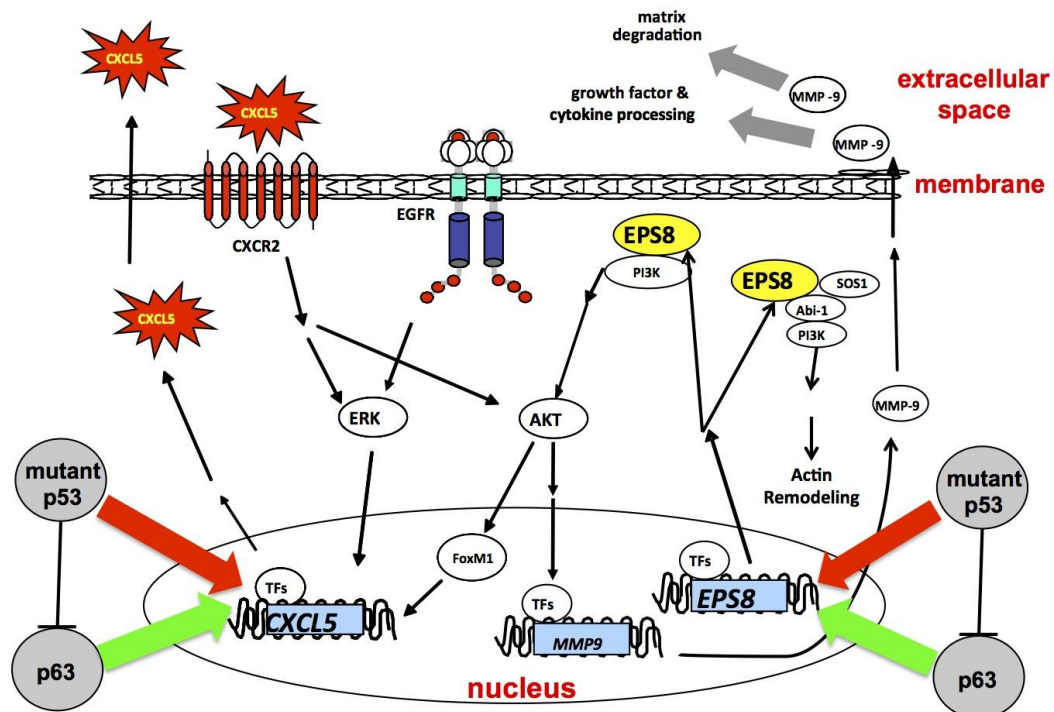


Figure 20 Proposed Relationships and Mechanism. We have shown a role for GOF mutant p53 enhancing EPS8 and CXCL5 expression by upregulating promoter activity in HNSCC (red). We also found evidence for an inhibitory role of p63, specifically $\Delta Np63$, on EPS8 and CXCL5 expression by downregulating promoter activity (green).

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